Antimycobacterial Action of Thiolactomycin: an Inhibitor of Fatty Acid and Mycolic Acid Synthesis†

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Thiolactomycin (TLM) possesses in vivo antimycobacterial activity against the saprophytic strain *Mycobacterium smegmatis* mc²155 and the virulent strain *M. tuberculosis* Erdman, resulting in complete inhibition of growth on solid media at 75 and 25 µg/ml, respectively. Use of an in vitro murine macrophage model also demonstrated the killing of viable intracellular *M. tuberculosis* in a dose-dependent manner. Through the use of in vivo [1,2-¹⁴C]acetate labeling of *M. smegmatis*, TLM was shown to inhibit the synthesis of both fatty acids and mycolic acids. However, synthesis of the shorter-chain α'-mycolates of *M. smegmatis* was not inhibited by TLM, whereas synthesis of the characteristic longer-chain α-mycolates and epoxymycolates was almost completely inhibited at 75 µg/ml. The use of *M. smegmatis* cell extracts demonstrated that TLM specifically inhibited the mycobacterial acyl carrier protein-dependent type II fatty acid synthase (FAS-II) but not the multifunctional type I fatty acid synthase (FAS-I). In addition, selective inhibition of long-chain mycolate synthesis by TLM was demonstrated in a dose-response manner in purified, cell wall-containing extracts of *M. smegmatis* cells. The in vivo and in vitro data and knowledge of the mechanism of TLM resistance in *Escherichia coli* suggest that two distinct TLM targets exist in mycobacteria, the β-ketoacyl-acyl carrier protein synthases involved in FAS-II and the elongation steps leading to the synthesis of the α-mycolates and oxygenated mycolates. The efficacy of TLM against *M. smegmatis* and *M. tuberculosis* provides the prospects of identifying fatty acid and mycolic acid biosynthetic genes and revealing a novel range of chemotherapeutic agents directed against *M. tuberculosis*.

Much emphasis is being placed on the identification and manipulation of the targets of existing drugs as a rational approach to the development of new drugs against tuberculosis. Isoniazid (INH), the most widely used of these drugs, is highly selective against *Mycobacterium tuberculosis* (MIC, 0.2 µg/ml) and has been the subject of intensive research on its mode of action, culminating in evidence that its primary effect is on mycolic acid synthesis (36). However, most instances of resistance to INH are attributable to missense mutations in the *katG* gene (38), which encodes the KatG catalase-peroxidase enzyme responsible for the activation of INH to an anionic derivative (13, 14). More recently, the *inhA* gene was identified as the source of other instances of INH and ethionamide resistance, through point mutations in the gene itself or the 5' regulatory region (1), and the InhA protein has been shown to catalyze the NADH-specific reduction of medium-chain (C₁₂ to C₂₄) 2-*trans*-enoyl-acyl carrier protein (ACP) intermediates (27) involved in fatty acid elongation, consistent with its involvement in the early stages of fatty acid-mycolic acid biosynthesis. Mycolic acids [R₁-CH(OH)-CH(R₂)-COOH] are high-molecular-weight, α-alkyl, β-hydroxy fatty acids and are the most characteristic component of the cell walls of *Mycobacterium* and related genera (Fig. 1) (20). In the mycobacterial cell envelope, they are present in free lipids, mainly trehalose dimycolate (TDM) and trehalose monomycolate (20), and, for the most part, esterified to the terminal pentaarabinofuranosyl units of arabinogalactan and hence part of the backbone of mycobacterial cell walls (2, 18).

Thiolactomycin [(4R)(2E,5E)-2,4,6-trimethyl-3-hydroxy-

2,5,7-octatriene-4-thiolide] (TLM) is a unique thiolactone antibiotic isolated initially from a soil *Nocardia* sp. (24, 29) which exhibits potent in vivo activity against many pathogenic bacteria (21, 23). It is of relevance in the present context that TLM inhibits bacterial and plant type II fatty acid synthase (FAS-II) but not mammalian or yeast type I fatty acid synthase (FAS-I) (9); for instance, in *Escherichia coli*, TLM inhibits both β-ketoacyl-ACP synthase I to III and acetyl coenzyme A (CoA): ACP transacylase activities in vivo and in vitro (9, 11, 22, 32). Consequently, an understanding of the mode of action of TLM is important in the development of more effective antibiotics that exhibit selective action against bacterial FAS-II. The effects of TLM on mycobacterial multifunctional FAS-I, monofunctional mycobacterial polypeptide FAS-II, and the largely undefined mycolate synthase were investigated in the search for new antituberculosis drug targets and treatments of drug-resistant *M. tuberculosis*.

MATERIALS AND METHODS

Large-scale growth of mycobacteria. *M. smegmatis* mc²155 (which was obtained from William R. Jacobs, Albert Einstein College of Medicine of Yeshiva University, Bronx, N.Y.) was grown in 4-liter Fernbach flasks containing 2 liters of a glycerol-alanine-salts-based medium (31). Cells were grown to the mid-log phase (~36 h), harvested, washed with physiologically buffered saline, and stored at -70°C until required. Each 4-liter flask yielded approximately 2 g (wet weight) of cells.

Treatment of cultures with TLM. TLM was synthesized as described by Wang and Salvino (34). MICs were determined by the agar disk elution method (30). Briefly, cultures of *M. smegmatis*, *M. tuberculosis*, and *Corynebacterium matruchotti* (obtained from K. Takayama, University of Wisconsin, Madison) were diluted to 10³ to 10⁷ bacteria per ml, and an aliquot (0.1 ml) of each dilution was grown on plates of 7H11 medium (Difco Laboratories, Detroit, Mich.) containing the oleic acid-albumin-glucose dextrose (5), and 0, 1, 5, 25, 50, 75, 100, 150, or 200 µg of TLM per ml was added. The plates were incubated at 37°C for 4 days for *M. smegmatis* and 21 days for *M. tuberculosis*, and the number of viable bacteria was determined by counting colonies. The MIC was defined as the

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† Dedicated to the memory of Jason W. Armour, a young, inspiring colleague whose tragic death occurred after completion of this work.

FIG. 1. Structures of the major mycolic acids of *M. smegmatis* and *M. tuberculosis*.

lowest concentration of TLM required to produce a 99% endpoint, a conventional endpoint used to test antibiotics against *M. tuberculosis* (30).

Liquid culturing of *M. smegmatis* was conducted with 7H9 broth (Difco)–0.2% glycerol–0.05% Tween 80 at 37°C. At the late-log phase (A_{600} , ~1.0), cultures were inoculated (final A_{600} , ~0.1) in triplicate into 8.5-ml Teflon screw-cap tubes containing fresh medium and increasing concentrations of TLM (10, 25, 50, 75, 250, and 500 µg/ml in 1% ethanol). The cultures were then subjected to gentle agitation at 37°C for 4 days, and growth was monitored by measuring A_{600} .

Incorporation of [1,2- 14 C]acetate into fatty and mycolic acids. *M. smegmatis* was grown in the broth medium in 20 tubes to the mid-log phase, at which point TLM (75 µg/ml) was added to half of the tubes, followed by further incubation at 37°C for 1 h. [1,2- 14 C]acetate (Na salt; 110 mCi/mmol; American Radiolabelled Chemicals, Inc., St. Louis, Mo.) was added at 1 µCi/ml to both control and TLM-treated cultures which were further incubated at 37°C with gentle agitation for 16 h. The 14 C-labeled cells were harvested by centrifugation at $2,000 \times g$ and washed twice with 0.9% NaCl and once with water. The combined 14 C-labeled cells from both the control and TLM-treated cultures were divided into two sets of five, respectively.

Determination of effects of TLM on fatty acid and mycolic acid metabolism. One set of 14 C-labeled control and TLM-treated cells were subjected to alkaline hydrolysis in 15% tetrabutylammonium hydroxide at 100°C overnight, followed by the addition of 2 ml of CH_2Cl_2 and 100 µl of CH_3I (Aldrich Chemical Co., Milwaukee, Wis.). The entire reaction mixture was then mixed on a shaking table for 30 min and centrifuged, and the upper aqueous phase was discarded. The lower organic phase was then washed with dilute HCl, followed by water, and dried. A known aliquot (~100,000 cpm) of the resultant mixture of fatty acid methyl esters (FAMES) and α -, α' -, and epoxy-mycolic acid methyl esters (MAMES) were subjected to preparative thin-layer chromatography (TLC) on silica gel (5735 silica gel 60F₂₅₄; Merck, Darmstadt, Germany) and developed twice in petroleum ether-acetone (95:5) as previously described (35). Autoradiograms were produced by overnight exposure at –70°C to Kodak X-Omat AR film to reveal the 14 C-labeled FAME (R_f , 0.9) and α -, α' -, and epoxy-MAMES (R_f , 0.4 to 0.5), which were counted directly to estimate total synthesis and the degree of inhibition of synthesis of FAMES and MAMES by TLM.

Isolation of 14 C-labeled TDM and cell wall-bound mycolates. The second set of control and TLM-treated, 14 C-labeled *M. smegmatis* was used to examine the effects of TLM on the synthesis of free-mycolate-containing components, primarily TDM, and bound cell wall mycolates. Both 14 C-labeled control and TLM-treated cells were extracted with CHCl_3 – CH_3OH (2:1) to yield a lipid-soluble fraction which was further subjected to a biphasic Folch wash (6). The

lower organic phase, rich in TDM, was subjected to preparative TLC in CHCl_3 – CH_3OH – NH_4OH (80:20:2) and exposed to autoradiography film. The corresponding 14 C-TDM products were marked, extracted from the TLC plates, and counted (19). The residual 14 C-labeled, delipidated cells, rich in the bound mycolate esters of arabinogalactan, were subjected to alkaline hydrolysis, methylation, preparative TLC, autoradiography, and counting (35). In some instances, more complete resolution of the individual mycolate classes was achieved through two-dimensional silver ion argention TLC as previously described (8).

Preparation of soluble cytosolic and particulate cell wall enzyme fractions. *M. smegmatis* (30 g [wet weight]) was washed and resuspended in a buffer (30 ml) containing 100 mM potassium phosphate (pH 7.0), 1 mM EDTA, 5 mM dithiothreitol, 5 mM MgCl_2 , and 2 mM phenylmethylsulfonyl fluoride at 4°C and subjected to probe sonication (1-cm probe; Soniprep 150; MSE Ltd., Crawley, Sussex, United Kingdom) for 10 min in 10 60-s pulses with 90-s cooling intervals between pulses. The whole sonicate was centrifuged at $27,000 \times g$ for 30 min at 4°C, and the supernatant was recentrifuged at $100,000 \times g$ for 1 h to yield the soluble pale yellow cytosolic enzyme fraction with a typical protein concentration of 8 to 10 mg/ml and containing FAS-I and FAS-II enzyme activities. The particulate (P60) cell wall enzyme fraction with mycolate-synthesizing activity was prepared as described previously (35).

Assays for FAS-I, FAS-II, and mycolic acid-synthesizing activities. The standard reaction mixture for the incorporation of radioactivity from [2- 14 C]malonyl-CoA into C_{16} to C_{24} fatty acids catalyzed by FAS-I was composed as follows (3, 4): 100 mM potassium phosphate (pH 7.0), 5 mM EDTA, 5 mM dithiothreitol, 300 µM acetyl-CoA, 100 µM NADPH, 100 µM NADH, 1 µM flavin mononucleotide, 500 µM α -cyclodextrin, 20 µM malonyl-CoA, 100,000 cpm of [2- 14 C]malonyl-CoA, and 100 µl of the cytosolic enzyme preparation (1 to 2 mg of protein) in a total volume of 500 µl. Similarly, the standard reaction mixture for incorporation of radioactivity from [2- 14 C]malonyl-CoA into C_{24} to C_{30} fatty acids catalyzed by FAS-II contained the following (3, 4): 100 mM potassium phosphate (pH 7.0), 5 mM EDTA, 5 mM dithiothreitol, 100 µM palmitoyl-CoA, 140 µM NADPH, 140 µM NADH, 180 µg of ACP, 40 µM malonyl-CoA, 200,000 cpm of [2- 14 C]malonyl-CoA, and 100 µl of the cytosolic enzyme preparation (1 to 2 mg of protein) in a total volume of 500 µl. TLM (0.1 to 0.75 µg/ml) was added with the other assay components prior to the addition of protein. In both the FAS-I and FAS-II assays, reactions were performed in triplicate at each concentration of TLM at 37°C for 30 min and terminated by the addition of 500 µl of 20% potassium hydroxide in 50% methanol at 100°C for 30 min. Following acidification with 300 µl of 6 M HCl, the resultant 14 C-labeled fatty acids were extracted three times with petroleum ether. The organic extracts were pooled,

FIG. 2. Total synthesis of racemic TLM by the five-step procedure of Wang and Salvino (34) from the α -methyl- β -ketoester (compound 1). The key step involved addition of 3-ethoxy-2-methyl-2-propenal to the thiolactone (compound 4), followed by mild acid hydrolysis and subsequent treatment with methylene triphenylphosphorane to afford racemic TLM (compound 6). PyHBr₃, pyridinium bromide perbromide; CH₃COSH, thioacetic acid; NaH, BuLi, sodium hydride, butyl lithium; Ph₃PCH₂, methylene triphenylphosphorane.

washed once with an equal volume of water, and dried in a scintillation vial prior to counting. Incubations utilizing the mycolate-synthesizing P60 cell wall fraction were performed as described by Wheeler et al. (35). The reaction mixture contained 50 mM potassium phosphate (pH 6.0), 10 mM KHCO₃, 1 μ Ci of [1,2-¹⁴C]acetate, and 440 μ l of the P60 preparation (2.0 to 3.5 mg of protein) in a total volume of 1 ml. TLM (0.1 to 1.0 μ g/ml) was mixed with the other assay components prior to the addition of protein. Reaction mixtures, which were prepared in triplicate at each concentration, were incubated at 37°C for 1 h, following which the reactions were terminated by addition of 2 ml of 15% aqueous tetrabutylammonium hydroxide and the corresponding FAMES and MAMES were isolated and counted as described earlier.

Preparation and infection of bone marrow macrophages. Six- to eight-week-old female specific-pathogen-free C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and killed by cervical dislocation, and the femurs were aseptically removed. The marrow was flushed from the femurs with ice-cold Dulbecco's minimal essential medium supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 U of penicillin per ml, 100 μ g of streptomycin per ml, 250 ng of amphotericin B per ml, minimal essential medium nonessential amino acids, 10% fetal calf serum (Summit Biotechnologies Inc., Fort Collins, Colo.), and 10% L-929 fibroblast conditioned medium (sDMEM). The marrow plugs were disrupted by gentle pipetting, washed twice, and plated at 10⁶ cells per well on 24-well tissue culture treated plates (Falcon 3047; Becton Dickinson Labware, Lincoln Park, N.J.). After 48 h of incubation in a 37°C, 5% carbon dioxide, humid environment, the nonadherent cells were removed and new sDMEM was added. After a further 4 days, the cells were washed, antibiotic-free medium was added and the mixture was incubated for 2 more days. Eight days after plating, macrophages were infected with 10⁶ CFU of *M. tuberculosis* Erdman in 200 μ l of medium for 2 h. Macrophages were then extensively washed to remove extracellular bacteria and incubated in sDMEM containing TLM at 4, 8, or 16 μ g/ml for 6 days. To determine the level of infection, macrophages were incubated with 1 ml of distilled water with 0.04% Tween 80 (Sigma) to lyse the cells and release the ingested bacteria. From this initial 1 ml of water, three 10-fold dilutions were made and 0.1 ml from each dilution was plated on Middlebrook 7H11 medium and incubated for 21 days in an air-dry, 37°C incubator. The number of viable bacteria in each well was determined by counting the number of colonies resulting from each dilution. Several wells were lysed immediately after the initial infection both to determine the number of bacteria phagocytosed and to assess the amount of bacterial growth over time.

RESULTS

Related antagonists and synthesis of TLM. TLM belongs to a small group of antibacterial compounds which have recently been collectively termed the thiotetronic acids. A convenient five-step chemical synthesis leading to racemic TLM recently reported by Wang and Salvino (34) was used in this study. Briefly, the synthetic strategy (Fig. 2) involved bromination of methyl α -propionylpropionate (compound 1) with pyridinium bromide perbromide in acetic acid followed by treatment of compound 2 with thioacetic acid to yield the thioacetate (com-

pound 3) which, when treated with base, afforded the key thiolactone intermediate (compound 4). The dianion generated from compound 4, when reacted with 3-ethoxy-2-methyl-2-propenal followed by mild acid hydrolysis, yielded the aldehyde compound 5, which was subsequently treated with methylene triphenylphosphorane to afford racemic TLM (compound 6). Various spectroscopic (¹H nuclear magnetic resonance and electron impact mass spectrometry) data, along with the chromatographic behavior of TLM, were in agreement with those reported in the literature (29, 34). For instance, the ¹H nuclear magnetic resonance spectrum of TLM measured with a Bruker ACE-300 spectrometer in CDCl₃ revealed the following characteristic signals: nine methyl protons at δ 1.67 (3H, singlet), δ 1.68 (3H, singlet), and δ 1.75 (3H, singlet); four olefinic protons at δ 4.98 (1H, doublet, *J* = 10.7 Hz), δ 5.18 (1H, doublet, *J* = 17.3 Hz), δ 5.56 (1H, singlet), and δ 6.25 (1H, doublet, *J* = 10.9, 17.3 Hz); and a proton at δ 8.00 (1H, broad). The electron impact mass spectrum of TLM gave an ion at *m/z* 210, consistent with the proposed structure and formula weight of TLM (29, 34).

Determination of the MIC of TLM on solid media. The susceptibilities of *M. smegmatis* and *M. tuberculosis* to various concentrations of TLM (1 to 150 μ g/ml) were determined on 7H11 solid medium containing the oleic acid-albumin-dextrose complex supplement and were compared to published values for INH and ethionamide. The MICs of TLM against *M. tuberculosis* and *M. smegmatis* were 25 and 75 μ g/ml, respectively, compared to the published value of 10 μ g/ml for ethionamide against both species and 0.2 and 5 μ g/ml for INH (1), respectively. Interestingly, another actinomycete, *C. matrucchotti*, was resistant to TLM, resulting in a complete lawn of growth at up to 100 μ g/ml on solid medium. The MICs reported here for TLM against *M. smegmatis* and *M. tuberculosis* may be substantially lower, since a racemic mixture of TLM was used. Previously, 60% inhibition of spinach leaf fatty acid synthesis with 50 μ M TLM was observed in vitro (12), whereas a MIC of 3.8 μ M was observed by Yamada et al. (37), which they attributed in part to the use of the naturally derived TLM from *Nocardia* sp. strain 2-200. Similar observations were made in the context of studies on the inhibitory effect of TLM on fatty acid synthesis in *E. coli* by Jackowski et al. (11) and Hayashi et al. (9).

Effect of TLM on the growth of mycobacteria within bone marrow macrophages. Addition of TLM to macrophage cultures containing *M. tuberculosis* resulted in bacterial killing in a dose-dependent manner (Fig. 3). In the absence of TLM, viable bacteria grew from log₁₀ 5.38 to log₁₀ 5.59 over a 6-day period, while in the presence of TLM, not only was growth inhibited but there was a reduction in the initial inoculum, demonstrative of mycobactericidal activity in accord with previous reports of rapid bacteriostatic action (23). Whether TLM is directly responsible for the observed bactericidal activity is unclear, since it could compromise the integrity of the bacterial envelope, thereby potentiating macrophage bactericidal activity.

Selective effects of TLM on aspects of fatty acid-mycolic acid metabolism. The growth rate of *M. smegmatis* in the presence of increasing concentrations of TLM was monitored by measuring *A*₆₀₀ over a period of 4 days. The amount of TLM required for complete growth inhibition on solid media was a striking six to eight times higher than that necessary to achieve a corresponding effect in liquid medium. Approximately 50% inhibition of biomass production compared with that of the control was observed at 75 μ g of TLM per ml, and as a consequence, this subinhibitory concentration was chosen to study fatty acid and mycolic acid synthesis in whole cells. *M. smeg-*

FIG. 3. Bactericidal activity of TLM when added to *M. tuberculosis* Erdman-infected macrophages. Murine bone marrow-derived macrophages were infected with 10^6 bacteria for 2 h, extracellular bacteria were removed, and macrophages were incubated in various concentrations of TLM for 6 days. Macrophage lysates were then individually plated on agar to determine the numbers of viable bacteria remaining in wells. Each value plotted represents the mean of triplicate wells at each concentration. A TLM concentration of 15.6 $\mu\text{g/ml}$ resulted in a 2 log (1.97) reduction in viable bacteria.

matris was initially grown in liquid medium (A_{600} , 1.0), subcultured into 20 tubes each containing 5 ml of liquid medium (A_{600} , 0.1), and grown at 37°C for an additional 8 h before the addition of TLM to half of the tubes, which were then preincubated at 37°C for 1 h before the pulse-labeling of all tubes with $[1,2-^{14}\text{C}]\text{acetate}$ (1 $\mu\text{Ci/ml}$) for 16 h at 37°C. The cellular mycolic and fatty acids were released by saponification with 15% tetrabutylammonium hydroxide and esterified by using phase transfer catalysis, and their respective FAMES and MAMES were separated by TLC and counted, thereby providing a facile screen for the effects of TLM on fatty acid and mycolic acid synthesis.

In one of many identical experiments, it was observed that TLM at 75 $\mu\text{g/ml}$ induced strong inhibition of both fatty acid (ca. 80%) and mycolic acid (ca. 86%) synthesis, and in addition, there were distinct differences in the extent of inhibition of the synthesis of each of the individual classes of mycolates; the α -mycolates were almost completely inhibited (96%), whereas the α' -mycolates were only slightly inhibited (36%), and synthesis of the epoxymycolates also appeared to be inhibited, although because of their low abundance and susceptibility to mild acid, no precise quantitation was possible. A more complete resolution and quantitation of the degree of inhibition of the synthesis of these mycolate classes were achieved through two-dimensional TLC employing silver ion argentation in the second direction (Fig. 4). It was clear from this exercise that the synthesis of the long-chain (C_{74} to C_{83}) α ($\alpha_1[\text{cis}, \text{cis}]$, and $\alpha_2[\text{cis}, \text{trans}]$)-mycolates of *M. smegmatis* was strongly inhibited by TLM, whereas that of the shorter-chain (C_{60} to C_{66}) α' -mycolates was apparently not affected (Fig. 4). Synthesis of the minor component identified as x in Fig. 4, which corresponds to the minor (<2%) cyclopropanated product of the proximal *cis*-olefin of α_1 (8), and the epoxymycolate(s) also appears to be strongly inhibited by TLM. The

relative proportions of ^{14}C in these classes of mycolates ($\alpha_1/\alpha_2/\alpha'$) in control cultures were 42:39:19, compared with 7:13:80 in TLM-treated cultures. In a related time course experiment (results not shown), TLM had a profound effect within the first 30 min of exposure upon the synthesis of the α (both α_1 and α_2)- and α' -mycolates of *M. smegmatis*, resulting in a total reversal in the spectrum of the newly synthesized mycolates within 120 min.

Two distinct populations of covalently bound mycolic acids exist in mycobacteria (20), those extractable by organic solvents, mainly TDM, and those covalently bound to the terminal pentaarabinosyl units of the insoluble arabinogalactan-peptidoglycan complex (18). To examine the effect of TLM on those two populations, the remaining control and TLM-treated cultures from the above-described $[1,2-^{14}\text{C}]\text{acetate}$ labeling experiment were extracted first with $\text{CHCl}_3\text{-CH}_3\text{OH}$ (2:1) to remove $[^{14}\text{C}]\text{TDM}$. TLC, followed by autoradiography and scintillation counting of the prominent ^{14}C -TDM band, revealed that TLM at 75 $\mu\text{g/ml}$ caused a dramatic 73% decrease in TDM synthesis. Alkaline hydrolysis of the residual, delipidated cells, followed by methylation and analysis of the resulting MAMES, demonstrated 93% inhibition of ^{14}C incorporation from the $[1,2-^{14}\text{C}]\text{acetate}$ into the α -mycolate and 36% inhibition of ^{14}C incorporation into the α' -mycolates.

Selectivity of TLM inhibition of fatty acid and mycolic acid synthesis in mycobacteria. Mycobacteria are unusual in that they possess two FAS systems, multifunctional mycobacterial FAS-I and dissociated mycobacterial FAS-II (3, 4). Since TLM is a known inhibitor of only FAS-II β -ketoacyl-ACP synthases in *E. coli*, we postulated that the observed inhibition of fatty acid synthesis during the labeling of whole mycobacteria cells with $[1,2-^{14}\text{C}]\text{acetate}$ is due to selective inhibition of the dissociated FAS-II β -ketoacyl-ACP synthase. To test this hypothesis, the ability of TLM to selectively inhibit mycobacterial FAS-I in cell extracts from *M. smegmatis* was examined (Fig. 5A). The multifunctional mycobacterial FAS-I system was in-

FIG. 4. Two-dimensional silver ion argentation autoradiographic TLC of $[1,2-^{14}\text{C}]\text{acetate}$ pulse-labeled cells of *M. smegmatis* in the absence (A) and presence (B) of TLM. Control and TLM-treated cultures were grown to an approximate A_{600} of 0.3 before the addition of TLM at 75 $\mu\text{g/ml}$ and preincubated at 37°C for 1 h before the addition of $[1,2-^{14}\text{C}]\text{acetate}$ (1 $\mu\text{Ci/ml}$) to both control and TLM-treated cultures with further incubation-agitation for 16 h at 37°C. ^{14}C -labeled FAMES-MAMES were isolated as described in Materials and Methods, and ca. 100,000 cpm of both control and TLM-treated extracts was applied to two-dimensional silver ion argentation TLC plates. The plates were developed in the first direction by using two developments of hexane-ethyl acetate (95:5) and in the second direction by using a triple development of petroleum ether-diethyl ether (85:15). Autoradiograms were obtained after exposure to Kodak X-Omat AR film at -70°C for 16 h. Radioactive products were marked, extracted, and counted. The epoxy (e)-, α_1 -, α_2 -, and α' -MAMES and F-FAMES are indicated. The minor component x is the result of cyclopropanation of the proximal olefin of α_1 -MAME.

FIG. 5. Dose-response effect of TLM on FAS-I and FAS-II (A) and P60 mycolate-synthesizing activity of *M. smegmatis* in vitro (B). (A) Cytosolic enzymatic extracts were prepared from *M. smegmatis* and assayed for FAS-I and FAS-II activity by using [14 C]malonyl-CoA in the presence of TLM at 0.1, 0.25, 0.5, and 0.75 μ g/ml as described in Materials and Methods. The enzymatic reactions were performed in triplicate and terminated by addition of potassium hydroxide in methanol, and acidified, 14 C-labeled fatty acids were extracted with petroleum ether and counted. (B) Cell wall enzymatic extracts (P60) were prepared from *M. smegmatis* and assayed for mycolate-synthesizing activity in the presence of TLM at 0.1, 0.25, 0.5, 0.75, and 1.0 μ g/ml. The enzymatic reactions were performed in triplicate and terminated by addition of 15% tetrabutylammonium hydroxide and incubation at 100°C overnight, and the corresponding α - and α' -MAMEs were isolated and counted.

sensitive to TLM in a dose-dependent manner at up to 0.75 μ g/ml. However, a corresponding dose-response curve against the dissociated mycobacterial FAS-II system demonstrated 60% inhibition of the FAS-II synthase at 0.75 μ g/ml, presumably through its effect on the FAS-II β -ketoacyl-ACP synthase (Fig. 5A). Interestingly, formation of the medium-chain (C_{24} to C_{30}) fatty acids produced by FAS-II was much more sensitive to TLM inhibition in the in vitro [14 C]malonyl-CoA assay

than in the in vivo labeling experiments using [$^{1,2-14}$ C]acetate. A corresponding effect was also observed by Tsay et al. (32) during their work on the characterization of β -ketoacyl-ACP synthase I and the mechanism of TLM resistance in *E. coli* K-12.

For the effects of TLM on mycolic acid synthesis, we postulated a similar mechanism of action and an identical target. The synthesis of the long-chain α -mycolates and oxygenated mycolates probably occurs via a series of elongation steps from the intermediate α' -mycolate, involving, among other enzymes, a type II β -ketoacyl-ACP synthase. The introduction of oxygen functions probably occurs late in the pathway, and as a consequence, inhibition by TLM of the elongation steps leading to the α -mycolates would lead to subsequent inhibition of oxygenated mycolic acid synthesis. To test this possibility, cell-free, particulate cell wall enzyme (P60) extracts, which preferentially catalyze the synthesis of mycolates, were analyzed in a dose-response manner against TLM (Fig. 5B). As predicted, synthesis of the α' -mycolates was insensitive to TLM at up to 1.0 μ g/ml, whereas that of the α (both α_1 and α_2)-mycolates was strongly inhibited, resulting in approximately 65% inhibition at 1.0 μ g/ml (Fig. 5B). Medium-chain (C_{16} to C_{24}) fatty acids produced within the in vitro mycolate assay were not inhibited by TLM (results not shown). It should be noted that MAME formation was much more sensitive to TLM in the in vitro mycolate assay than in the in vivo labeling experiments described earlier.

DISCUSSION

Extensive studies on fatty acid biosynthesis in both prokaryotes and eukaryotes have resulted in the definition of two distinct classes of enzyme activities necessary for the synthesis of long-chain fatty acids (10, 17, 33). Many bacteria and plants possess a dissociable FAS-II, where each individual reaction is catalyzed by a discrete monofunctional peptide. In contrast, yeast and mammalian systems possess a multifunctional FAS-I. The extensive work of Bloch demonstrated that *M. smegmatis* is unique in that it possesses both the multifunctional mycobacterial FAS-I and the monofunctional mycobacterial polypeptide FAS-II systems (3, 4). The primary products produced by the de novo FAS-I system were C_{16} to C_{18} and C_{24} to C_{26} fatty acyl-CoA derivatives, and the distribution of these products was bimodal and influenced by the presence of the mycobacterial 6-*O*-methylglucose- and 3-*O*-methylmannose-containing polysaccharides (25). The type I complex appears to have limited elongation capacity (3, 4) and is apparently not capable of extending fatty acids to the meromycolate level. The ACP-requiring FAS-II system was regarded as an elongation mechanism devoted primarily to the elongation of C_{16} fatty acid primers to C_{24} to C_{30} fatty acyl-ACPs, but again, this elongation apparently did not extend to the meromycolate level. Thus, it appears that the synthesis of mycolates, or at least meromycolates, requires a separate synthase. Qureshi et al. (28) did demonstrate elongation of a C_{24} fatty acid to C_{52} meromycolate, but not to mycolate, by a cell extract of *M. tuberculosis* H37Ra. More recently, a particulate cell-free system of *M. aurum* and *M. smegmatis* was developed which allowed the synthesis of whole mycolic acids from acetate rather than malonate (15, 35) and also from a $C_{24:1}\Delta 5$ fatty acid intermediate.

The issues that are most relevant in the present context concern the mode of action of TLM in terms of the mycobacterial FAS-I and FAS-II synthases and how this relates to the action of TLM, e.g., on the *E. coli* FAS-II synthase (32), and also the question of the utility of TLM as an example of a new

generation of antagonists targeted against mycolic acid synthesis and mycobacteria. In general terms, there are two accepted pathways for the initiation of fatty acid biosynthesis in *E. coli* (10, 17). Firstly, acetyl-CoA is transacylated to ACP via the acetyl-CoA:ACP transacylase and then condensed by either β -ketoacyl-ACP synthase I or II with malonyl-ACP to form acetoacetyl-ACP. The second route to acetoacetyl-ACP is the condensation of acetyl-CoA with malonyl-ACP catalyzed by synthase III. In *E. coli*, TLM has long been considered to disrupt fatty acid biosynthesis by inhibiting the acetyl transacylase step (11) and all three β -ketoacyl-ACP synthases (22). However, a recent communication by Lowe and Rhodes (16) which described the purification of the acetyl-CoA:ACP transacylase to homogeneity demonstrated that this activity was not inhibited by TLM, and the extensive work of Rock and colleagues (17, 32) suggests that the β -ketoacyl-ACP synthase I may be the only activity required for the initiation of fatty acid biosynthesis and is the primary site of action of TLM in *E. coli*.

The monofunctional mycobacterial polypeptide FAS-II is unusual in that it functions primarily as an elongating system in mycobacteria (3, 4). The initial steps involved within this synthase, which are presumably analogous to the initiation steps in *E. coli* (11, 17), involve a palmitoyl-CoA:ACP transacylase to afford palmitoyl-ACP, which is then condensed with malonyl-ACP to yield the corresponding β -ketoacyl-ACP. The next step involves reduction of the β -ketoester-ACP by an NADPH-dependent β -ketoacyl-ACP reductase, followed by dehydration (β -hydroxyacyl-ACP dehydratase) to the *trans*-2-unsaturated acyl-ACP, which is then reduced by an NADH-dependent enoyl reductase to form the C_{18} ACP, which can then serve as a substrate for additional rounds of elongation. The *in vitro* FAS-I-FAS-II data presented in this communication demonstrate that TLM is a potent inhibitor of the monofunctional mycobacterial polypeptide FAS-II system, whereas the multifunctional mycobacterial FAS-I system is insensitive. This is consistent with other reports concerning the mode of action of TLM against mammalian and yeast FAS-I systems (9) and bacterial and plant FAS-II systems (22). In addition, through precedence with the FAS-II system of *E. coli*, the presumed target within the ACP-dependent monofunctional mycobacterial polypeptide FAS-II system would be a β -ketoacyl-ACP synthase (32). Interestingly, the related actinomycete species *C. matruchotti*, which possess only a closely related multifunctional FAS-I system (4) and synthases which are responsible for the synthesis of corynomycolates (C_{28} to C_{36}), was unsuspceptible to TLM on solid media. This is not surprising, since this study has defined the primary targets of TLM as two distinct β -ketoacyl-ACP synthases within mycobacteria, firstly, the ACP-dependent mycobacterial FAS-II system, and secondly, the synthases responsible for long-chain mycolate synthesis, neither of which is present in corynebacteria (Fig. 6). On the other hand, the observed insensitivity of corynebacteria to TLM may be due to other resistance mechanisms (7).

A recent report by Quemard and colleagues (27) suggested that InhA, which is a long-chain (C_{12} to C_{24}) enoyl-ACP-dependent reductase, is part of the type II ACP-dependent FAS-II system described by Bloch (3, 4). However, in our studies, INH in the presence of catalase-peroxidase activity (26, 38) and crude cell extracts of *M. smegmatis*, failed to inhibit the ACP-dependent FAS-II system described by Bloch (3, 4). The relevance of this observation to the present study becomes more obvious when the mode of action of TLM is considered in the context of FAS-II and the synthases responsible for mycolate synthesis. If the ACP-dependent FAS-II system were involved in the early stages of mycolic acid syn-

FIG. 6. TLM and mycolic acid biogenesis. The sites of action of TLM are represented by *thmA* and *thmB*. A third site of action, *thmC*, is involved in the synthesis of medium chain length fatty acids by the FAS-II elongation system described by Bloch (3, 4). The oxygenated mycolates include epoxy, keto, methoxy, and wax ester mycolates typical of *M. smegmatis*, *M. tuberculosis*, and *M. avium*. The α' -mycolates are representative of the monounsaturated mycolates of *M. smegmatis*.

thesis as suggested (27), then TLM, which has been shown to be a potent inhibitor of this system, would lead to the subsequent inhibition of all types of mycolic acids. However, this is not the case, since the α' -mycolates of *M. smegmatis* are still synthesized, while TLM exhibits selective inhibition of the ACP-dependent FAS-II system and an elongation step specific to the synthesis of the long-chain α -mycolates and oxygenated mycolates of *M. smegmatis* and *M. tuberculosis*. This suggests an alternative mechanism, presumably involving a membrane-bound ACP-dependent FAS-II system, of which InhA is a part, which performs *de novo* synthesis and provides fatty acid primers used exclusively for mycolate synthesis. In addition, this specialized type II mycolic acid synthase is particulate in nature, as demonstrated by its location within P60 extracts, and is clearly distinct from the soluble-ACP-dependent FAS-II system. Indeed, addition of INH to P60 extracts in the presence of catalase-peroxidase activity results in strong (>85%) inhibition of mycolate synthesis in *M. smegmatis* at 100 μ g/ml.

The other potential TLM target identified in this study was a key elongation enzyme, leading from either the $C_{24:1}\Delta 5$ intermediate or from the short-chain α' -mycolates to the long-chain α -mycolates and oxygenated mycolates of *M. smegmatis* and *M. tuberculosis*, presumably another β -ketoacyl-ACP synthase. The findings presented within this study, along with the isolation of InhA (1, 27), suggest that multiple ACP-dependent FAS-II systems exist involving multiple, unique β -ketoacyl-ACP synthases, β -ketoacyl-ACP reductases, β -hydroxyacyl-ACP dehydratases, and β -enoyl-ACP reductases involved in both fatty acid and mycolic acid synthesis. Each isozyme is apparently based on individual substrate specificities and therefore makes a unique contribution to the regulation and distribution of products from each pathway. However, direct evidence for their existence is not available because of a lack of adequate biochemical and genetic tools. Nevertheless, the identification of two TLM targets within *M. smegmatis* allows the generation of mutants and transformants capable of conferring TLM resistance either by enzyme overproduction or via point mutation leading to the identification of the genes in-

volved in TLM resistance and hence fatty acid and mycolic acid biosynthesis. Finally, it should be noted that other mechanisms of TLM resistance may exist in mycobacteria. For instance, in *E. coli*, disruption of the *emrB* gene restored sensitivity to TLM-resistant strain *E. coli* CDM5 (7) and we have demonstrated that overexpression of the *emrAB* operon confers TLM resistance on susceptible *E. coli* strains (7).

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